様式第１号

論文内容要旨

The crystal structure and oligomeric form of *Escherichia coli* L,D-carboxypeptidase A

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1. Introduction

Bacteria are surrounded by a rigid cell wall, which gives them their shape and protects them from rupture due to osmotic pressure. An important component of the cell wall is peptidoglycan (PG), a net-like structure consisting of strands of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNac) molecules connected by short peptide chains (Figure 1).

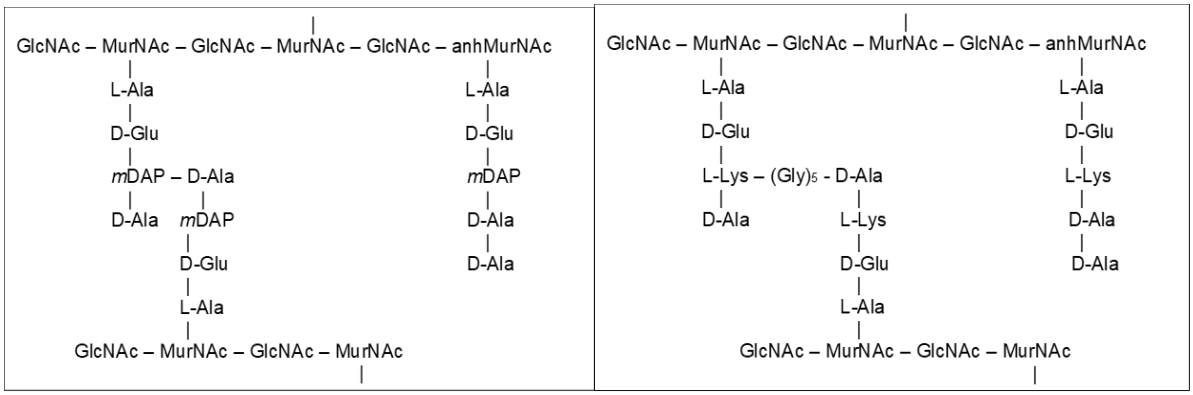

Figure 1: Peptidoglycan components of gram-negative bacteria (left) and gram-positive bacteria (right). The peptide stem of gram-negative bacteria is directly connected between amino acids at position 3 of one chain to position 4 of the other chain. Peptidoglycan of gram-positive bacteria is linked by a pentaglycine.

Whereas gram-negative bacteria have only few layers of PG and are covered by an outer membrane, gram-positive bacteria have a much thicker PG as the outmost layer. The connecting peptide stems contain unique D-amino acids, but differ in their sequence depending on the species. PG is constantly remodeled during growth and numerous hydrolases cleave PG, releasing the fragments to the medium or recover and recycle them. In the latter case the di-saccharide blocks with a tetra-peptide stem are imported back to the cytoplasm, further degraded and used in the synthesis of new PG (1). One enzyme in this cell wall recycling (*) pathway, the cytoplasmic L,D-carboxypeptidase A (LdcA), cleaves the terminal D-Ala from the tetra-peptide, which is essential since only the tripeptide can be reused. LdcA is the only enzyme for this task. Though there are differences in the recycling

process of gram-positive and gram-negative bacteria, LdcA homologs have been found in both types (2).

In the past antibiotics targeted mainly steps in the synthesis of PG, however due resistance towards these kind of antibiotics it is necessary to find new ways to fight bacterial infections. Recycling is a common way for bacteria to save resources and as much as 50% of PG are recycled in *E.coli* (3). LdcA is therefore a potential drug target.

I determined the structure of LdcA of the gram-negative *Escherichia coli* (*Ec*LdcA) in the native form as well as of an active site mutant to elucidate its mechanism. Further the oligomeric form (*) of the enzyme has been confirmed and the structure compared to the LdcAs of other species. Though it was not possible to obtain a structure with a bound ligand, the findings should help to lead to inhibitors in the cell wall recycling process.

2. Material and Methods

Expression and purification

The *ldcA* gene from *E. coli* was amplified by PCR from genomic DNA and inserted into pET28b plasmid between the restriction sites Nde I and Bam H1. The resulting plasmid was transformed into *E. coli* BL21 and grown in LB medium with 20 μg/ml kanamycin at 37℃ with shaking. When the OD_{600} reached 0.6, expression of the protein was induced by adding IPTG to a final concentration of 0.5 mM, and cell culture was continued at 20℃ overnight. The cells were collected, re-suspended and lysed by sonication. After centrifugation the supernatant was loaded on a nickel NTA agarose column, washed and then eluted with 250 mM imidazole. The dialyzed and filtered fractions were further purified using a HiTrap Q HP column and LdcA eluted at about 150 mM NaCl. Finally the protein was loaded onto a Superdex 200 gel filtration column equilibrated with 50 mM Tris-HCl pH 8.5, 50 mM NaCl. The main fractions were pooled and concentrated to 11 mg/ml for crystallization.

To introduce a site-directed mutation, appropriate primers were used to change the active site serine to alanine (S106A). The PCR product was digested with NdeI and BamH1 at 37℃ for 3 hours, purified and then ligated into a cut vector using T4 DNA ligase at 16℃ for 3 hours. This vector was transformed into *E. coli* DH5α cells and the plasmid with the mutant prepared with Nippon Genetics Miniprep Kit. Expression and purification were done by the same protocol as the native LdcA.

Crystallization and data collection

Using the hanging drop vapor diffusion method, crystals were grown in 0.02 M Tris-HCl pH 8.5, 0.2 M sodium thiocyanate and 12% PEG 3350. The space-group was I222. Data were collected at beamline BL17 at Photon Factory, Tsukuba to a resolution of 1.75 Å. Another crystallization condition was 85 mM HEPES pH 7.5, 3.655 M NaCl and 15% glycerol. These crystals were used for soaking in 10 mM heavy atom compound sodium ethylmercurithiosalicylate (EMTS) for 1 hour resulting in a usable derivative. Mercury-derivative data sets were collected at beamline BL-5A of the Photon Factory to resolution limits of 2.54 Å. Crystals of the LdcA mutant grew in 85 mM HEPES pH 7.5, 3.655 M NaCl and 15% glycerol, however in another space group $P2_12_12$. Data were collected at beamline BL17 at the Photon Factory, Tsukuba. The maximum resolution was 1.75 Å.

Structure determination

All data were integrated and scaled using the program XDS. Autosol, part of the Phenix suite, was used for experimental phase determination of the heavy-atom-soaked crystal data and the initial structure was built using Autobuild. This model was manually adjusted with COOT and refined with REFMAC. The resulting model was used as a template for molecular replacement of the high-resolution data of the native crystal. Model building and refinement were performed as described above. The structure of the mutant was determined by molecular

replacement using the native data as a template. Table 1 shows data collection and refinement statistics for the native and mutant models.

Data Set	Native PDB 5Z01	S106A mutant PDB 5Z03
Resolution range (Å)	46.9 – 1.75	46.7 – 1.75
Space group	*I*222	*P*2$_1$2$_1$2
Unit cell dimensions (Å)	*a*=77.9 *b*=89.0, *c*=93.8	*a*=68.8 *b*=93.4, *c*=86.8
Reflections (Measured/Unique)	300,303 / 33,235	363,628 / 56,189
Completeness (%)	99.9 / 99.9	99.1 / 85.0
Mean <*I*> / <σ(*I*)>	19.4 / 2.2	11.2 / 2.0
Multiplicity	9.0 / 9.3	6.5 / 5.5
*R*merge (%)[a]	7.3 / 101.6	9.7 / 83.2
B factor from Wilson plot (Å^2)	23.5	19.9
cc1/2 (%)	99.9 / 88.9	99.9 / 49.1
Refinement Statistics		
Resolution range (Å)	46.9 – 1.75	46.7 – 1.75
R-factor[b] / free *R*-factor (%)	17.9 / 22.5	22.9 / 27.0
Rms deviations from ideals		
bond lengths (Å) / bond angles (°)	0.02 / 1.80	0.019 / 1.90
No. of water atoms	212	56
B-values (all atoms, Å^2)		
protein/water	30.0 / 34.5	33.4 / 27.7
Ramachandran plot		
residues in most favorable regions	293	533
residues in allowed regions	10	35
residues in outlier regions	0	1

Values in outer shell are for the highest shell with resolution limits of 1.79-1.75 Å.
[a]*R*merge =Σ | I_i - <*I*> | / Σ | I_i | , where I_i is the intensity of an observation and <*I*> is the mean value for that reflection and the summations are over all reflections. Free R-factor was calculated with 5% of the data.
[b]*R*-factor =Σ $_h$||*Fo*(*h*)| - |*Fc*(*h*)|| / Σ$_h$*Fo*(*h*), where *Fo* and *Fc* are the observed and calculated structure factor amplitudes, respectively.

Table 1: Data collection and structure refinement statistics

Analytical Ultracentrifugation

To confirm that LdcA exists as a dimer in solution sedimentation velocity experiments were carried out. Sample and reference solution were loaded into a two-channel center-piece with sapphire windows. The rotor was kept stationary in the vacuum chamber for 1 hour for temperature equilibration. Absorbance scans at 280 nm were taken at 10 min. intervals during sedimentation at 30,000 rpm. The scans were analyzed using the continuous distribution module of the program SEDFIT. This distribution was converted to molar mass distribution.

3. Results

Crystallization

The LdcA protein crystallized under two different conditions and in different space groups. Though the original native crystal diffracted to a high resolution under the Tris-condition, crystals of the other condition (HEPES) were more robust towards soaking with heavy atoms. 5 mercury sites could be identified and the experimental phases were determined by SAD. The mutated LdcA was also crystallized under the HEPES condition and diffracted well, however the space group was an orthorhombic $P2_12_12$ with 2 molecules in the asymmetric unit (ASU), whereas the native crystal was I222 with 1 molecule in the ASU.

Overall structure

The *Ec*LdcA structure consists of 8 α-helices and 2 β-sheets. One of the β-sheets is on the surface and twisted, the other is between the helices 6 and 7 (Figure 2). Comparing the *Ec*LdcA structure to the LdcA of other gram-negative bacteria like *Pseudomonas aeruginosa* (*Pa*LdcA) (4) or *Novosphingobium aromaticivorans* (*Na*LdcA) (5) reveals that the structures have highly conserved parts, especially around the active site. Since the structures of both bacteria lack completeness, the closest related structure was of the self-immunity protein MccF, which cleaves the antimicrobial compound microcin C7 (6). Both molecules possess the same serine protease and both models align very closely (Figure 3). One difference is an extended loop to the active site in MccF of 29 residues, which corresponds to just 3 residues in *Ec*LdcA. It might be important for substrate specificity.

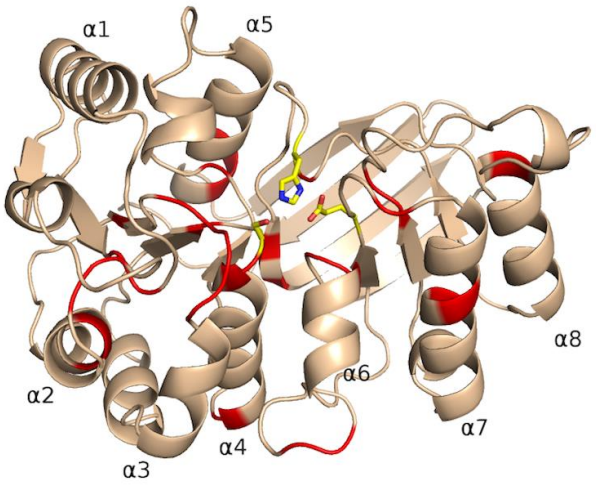

Figure 2: Ribbon diagram of one subunit of EcLdcA with conserved areas in red and the catalytic triad as sticks.

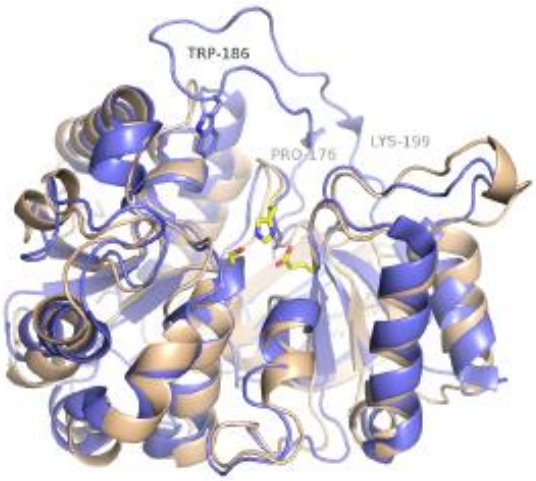

Figure 3: Overlay of ribbons of EcLdcA (beige) and MccF (blue) subunits

Active Site

The active sites of the LdcA structures of all four compared structures are highly conserved. All possess the serine protease with the catalytic triad (*) serine, glutamate and histidine (S106, E200, H270 in *Ec*LdcA).

Mutant

The mutant with the replacement of the active site serine by an alanine (S106A) was created to facilitate binding, but not cleavage of the substrate. However the missing serine hydroxyl group might have been responsible that the crystal crystallized under a different space group $P2_12_12$, although the crystallization condition was unchanged. The crystal diffracted to a maximum resolution of 1.75 Å and has a dimer in the asymmetric unit. 283 residues were ordered, however some residues were missing, but are all distant from the active site (>10 Å). The mutated active site itself does not show deviations due to the mutation. However, Gly75 and Gly76, both highly ordered in the native structure are more flexible in the mutant. The residue Ile202 differs considerably from the native

model. In the native LdcA its function is probably to keep His270 of the catalytic triad in place. This is not the case in the mutant, which is more flexible around these residues.

Oligomeric Form

The analytical ultracentrifugation experiment clearly showed, that *Ec*LdcA exists as a dimer in solution. There was only one clear peak at 69 kDa, indicating the dimer. Also the other models show a dimeric structure and all have a conserved arginine residue (Arg211 in *Ec*LdcA) pointing to the active site of the other subunit chain. Because it is rather close, it might have the function to hold or stabilize the substrate. Unfortunately a substrate-bound structure could not be obtained for confirmation in *Ec*LdcA.

*Ec*LdcA possesses a rather large dimer interface of about 1,700 Å with 48 contact points including salt bridges.

4. Discussion

The structures of 3 L,D-carboxypeptidases A in gram-negative bacteria revealed highly conserved areas especially around the active site. The recycling pathway requires tripeptides for re-use in PG synthesis, therefore an L,D-carboxypeptidase is essential to cleave the terminal D-Ala after re-import to the cytoplasm. To obtain a substrate-bound structure the commercially available pentapeptide was used. Purified DacB, a D-Alanine carboxypeptidase, responsible for cleaving the D-Ala on position five of the peptide stem at the transpeptidation step was utilized to create the substrate for LdcA, the tetrapeptide. However a substrate-bound structure could not be obtained neither by co-crystallization nor by soaking of LdcA crystals in the tetrapeptide solution.

Also in gram-positive bacteria with their much thicker PG layer recycling has been observed and LdcA homologs exist for some species. However structure and the mode of action differ considerably from gram-negative bacteria.

5. Summary

The structure of *E. coli* LdcA peptidase was solved in the native form as well as with an active site mutant replacing serine by alanine. This mutated structure showed an increased flexibility at the active site. Other bacterial species show similar conserved features of their LdcA active sites. A substrate-binding structure could not be obtained, indicating a strong specificity towards the substrate. The oligomeric form was confirmed to be a homodimer.

6. Publication List

Karen Meyer, Christine Addy, Satoko Akashi, David I. Roper, Jeremy R.H. Tame; The crystal structure and oligomeric form of Escherichia coli L,D-carboxypeptidase A. Biochemical and Biophysical Research Communications, (2018); in press

7. References

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2) Jan Reith and Christoph Mayer; Peptidoglycan turnover and recycling in gram-positive bacteria. Appl Microbiol Biotechnol, 92, 1-11 (2011)
3) James T. Park and Tsuyoshi Uehara; How Bacteria Consume Their Own Exoskeletons (Turnover and Recycling of Cell Wall Peptidoglycan). Microbiology and Molecular Biology Reviews, 211-227 (2008)

4) Henryk J. Korza and Matthias Bochtler; *Pseudomonas aeruginosa* LD-Carboxypeptidase, a Serine Peptidase with a Ser-His-Glu Triad and a Nucleophilic Elbow. The Journal of Biological Chemistry, Vol. 280, No. 49, 40802-40812 (2005)
5) Debanu Das, Mireille Herve, Marc-Andre Elsliger, Rameshwar U. Kadam, Joanna C. Grant, Hsiu-Ju Chiu, Mark W. Knuth, Heath E. Klock, Mitchell D. Miller, Adam Godzik, Scott A. Lesley, Ashley M. Deacon, Dominique Mengin-Lecreulx and Ian A. Wilson; Structure and Function of a Novel ld-Carboxypeptidase A Involved in Peptidoglycan Recycling. Journal of Bacteriology, Vol. 195, No.24, 5555-5566 (2013)
6) Vinayak Agarwal, Anton Tikhonov, Anastasia Metlitskaya, Konstantin Severinov and Satish K. Nair; Structure and function of a serine carboxypeptidase adapted for degradation of the protein synthesis antibiotic microcin C7. PNAS, 109(12), 4425-4430 (2012)

8. Glossary

Cell-wall recycling: bacteria carefully fragment their cell wall avoiding lysis, but enabling insertion of new PG. Only a small part of the fragments are released, most are recovered and reused for several reasons: saving resources, suppression of host immune response, induction of β-lactamase production etc.

Catalytic triad: Three amino acids, which are spatially close in the folded enzyme (not necessarily in sequence) that work together to hydrolyse a substrate. A nucleophile (serine in the case of LdcA) attacks the substrate. A histidine and acidic residue act to alter the pK of the serine, to make it a better nucleophile.

Oligomeric form: is the quaternary structure of a protein consisting of more than one subunit, which self-assemble and are not covalently bond. Oligomerization has usually structural and functional advantages for proteins.